Roles of endogenous cytokines in liver apoptosis of mice in lethal Listeria monocytogenes infection

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Abstract

Various bacterial pathogens have been identified as mediators of apoptosis. Apoptosis reportedly shows both detrimental and beneficial effects on biological functions. We studied the role of liver apoptosis in lethal Listeria monocytogenes infection and the regulation of apoptosis by endogenous cytokines during infection. Apoptosis was observed in the spleen but not in the liver of infected mice, whereas the induction of liver necrosis was evident by rising levels of serum aminotransferases in these animals. Apoptosis was detected in the liver of L. monocytogenes-infected mice which had been treated with monoclonal antibody (mAb) against tumor necrosis factor-α (TNF-α) or interleukin-6 (IL-6), or in TNF-α−/− mice, but not in γ interferon (IFN-γ)−/− mice or mice which had been treated with mAb against IL-4 or IL-10. Augmentation of liver apoptosis in mice treated with mAb against TNF-α or IL-6 or in TNF-α−/− mice correlated with the increase in bacterial numbers in the organ, while no augmentation of apoptosis was observed in the liver of IFN-γ−/− mice irrespective of the marked increase in bacterial numbers in the organs, indicating that augmentation of liver apoptosis may not be merely due to the increase in bacterial growth in the organs. These results suggest that TNF-α and IL-6 may play an important role in protecting the liver from apoptosis in lethal L. monocytogenes infection. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Listeria monocytogenes; Apoptosis; Tumor necrosis factor-α; γ-Interferon; Interleukin-6

1. Introduction

Apoptosis is a naturally-occurring mechanism of cell death involved in a large range of physiological as well as pathological events and is characterized by a set of specific alterations in cell morphology. Various bacterial pathogens have been identified as mediators of apoptosis [1,2]. These pathogens infect many different tissues and include Gram-positive and Gram-negative bacteria. Apoptosis shows both detrimental and beneficial effects on biological functions [3]. Pathogens could escape from a host-defense system by killing professional phagocytes and lymphocytes through apoptosis, whereas intracellular pathogens may lose their habitat by apoptosis. 

Listeria monocytogenes is a facultative intracellular-growing bacterium and murine listeriosis is an excellent experimental model used extensively to study the factors that affect host resistance to systemic bacterial infections [4]. L. monocytogenes cells inoculated intravenously (i.v.) into mice are eliminated rapidly from the bloodstream and most are taken up in the liver [5,6]. Most organisms taken up are bound extracellularly by hepatic cells and subsequently killed by immigrating neutrophils [6]. The bulk of L. monocytogenes cells that escape from the antibacterial
activity of neutrophils are internalized by the parenchymal cells, hepatocytes, which are the principle site of bacterial replication in the liver of mice [5,6]. Liver injury is observed in severe infection [7] and apoptotic hepatocytes are reportedly observed [8].

Studies on experimental models of liver injury have shown that tumor necrosis factor-α (TNF-α) plays a central role in liver failure concomitant with endotoxin shock in β-galactosamine-sensitized mice [9,10] and with fulminant hepatitis induced by bacterial lipopolysaccharide (LPS) in Propionibacterium acnes-sensitized mice [11]. Moreover, γ-interferon (IFN-γ) is involved in the induction of hepatitis induced by LPS in P. acnes- or Bacillus Calmette-Guérin (BCG)-sensitized mice [12,13], or by concanavalin A (Con A) in mice [14]. Apoptotic death of hepatocytes has been observed to occur concomitantly in these systems and is reportedly regulated by TNF or IFN-γ [11,14–16].

Cytokines such as IFN-γ, TNF-α and interleukin-6 (IL-6) are produced in the bloodstream and in organs including the liver and spleen in nonlethal and lethal infections with L. monocytogenes [17,18], and they play important roles in antilisterial resistance [9,10,19–22]. We investigated the role of liver apoptosis in lethal L. monocytogenes infection and the regulation of apoptosis by endogenous cytokines during infection. In this study, we demonstrated that endogenous TNF-α and IL-6 appear to play an important role in protecting the liver from apoptosis in lethal L. monocytogenes infection, whereas IFN-γ may not be involved in the regulation of liver apoptosis.

2. Materials and methods

2.1. Animals

The following mice used in this study were all 5–8 weeks old: outbred ddY mice, C57BL/6 mice, IFN-γ-deficient (IFN-γ−/−) mice on a C57BL/6 × Sv129 [14], and TNF-α-deficient (TNF-α−/−) mice on a C57BL/6 × Sv129 [23]. Female ddY mice and C57BL/6 mice were purchased from SLC Japan (Hamamatsu, Shizuoka, Japan). The animals were maintained under specific pathogen-free conditions at the Institute for Animal Experiment, Hirosaki University School of Medicine.

2.2. Bacteria

L. monocytogenes 1b-1684 cells [24] were grown in tryptic soy broth (Difco Laboratories, Detroit, MI, USA) at 37°C for 16 h. Mice were infected i.v. with 0.2 ml of a solution containing 5 × 10⁸ colony-forming units (cfu) of viable L. monocytogenes 1684 cells in 0.01 M phosphate-buffered saline (PBS; pH 7.4). L. monocytogenes 1684 had a 50% lethal dose for ddY mice of 2 × 10⁴ cfu and that for C57BL6 of 2 × 10⁵ cfu.

2.3. Depletion of endogenous cytokines

Hybridoma cell lines secreting monoclonal antibodies (mAbs) against mouse IFN-γ (R4-6A2; rat IgG1), mouse TNF-α (MP6-XT22.11; rat IgG1), mouse IL-4 (11B11; rat IgG1), mouse IL-6 (MP5-20F3.11; rat IgG1) and mouse IL-10 (JES5-2A5.11; rat IgG1) were injected into pristane-primed CD-1 nude mice. The mAbs found in the ascites fluid were partially purified by 50% (NH₄)₂SO₄ precipitation. Mice were given a single 1-mg dose by i.v. injection of each mAb 1 h before infection. Normal rat globulin (NRG) was injected as a control for the mAbs. NRG was prepared as described previously [24].

2.4. Determination of numbers of viable L. monocytogenes cells in the organs

The number of viable L. monocytogenes cells in the liver and spleen of infected mice was established by plating serial 10-fold dilutions of organ homogenates in PBS on tryptic soy agar (Difco). Colonies were routinely counted 18 to 24 h later.

2.5. Measurement of serum aminotransferases

Serum samples were diluted 1:5 with distilled water, and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured with a clinical Analyzer 7600 (Hitachi Medico, Hitachi, Ibaraki, Japan).

2.6. Analysis of DNA fragmentation

A small piece (0.05 g) of the liver was homogenized in 600 μl of cold lysis buffer containing of 10 mM Tris–HCl (pH 8.0), 20 mM EDTA (pH 8.0) and 0.5% Triton X-100. The lysates were microcentrifuged at 12,000 × g for 15 min at 4°C and the supernatants which contained DNA with low molecular mass were obtained. DNA in the supernatant was extracted twice with phenol–chloroform and once with chloroform and then precipitated by the addition of 1 ml of ethanol and 13 ml of 5 M NaCl and kept for 1 h at −80°C. DNA was sedimented by centrifugation at 7000 × g for 5 min and washed with 500 μl of 75% ethanol. After DNA was pelleted and dried, samples were incubated with 25 μl of RNase A (0.25 mg ml⁻¹, Sigma, St. Louis, MO, USA) overnight at 37°C. Aliquots were then electrophoresed in 3% agarose gels in Tris–acetate EDTA buffer (40 mM Tris, 40 mM acetic acid and 1 mM EDTA). The gel was stained with ethidium bromide and the DNA fragments were visualized under ultraviolet light.

2.7. Histology

NRG-, anti-TNF-α mAb- or anti-IL-6 mAb-treated mice were infected with 5 × 10⁵ cfu of L. monocytogenes. The liver tissues were removed 48 h later and were fixed

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for 24 h in 4% paraformaldehyde, dehydrated in graded ethanol and embedded in paraffin. Paraffin sections of 4 μm were adhered to slides pretreated with a 0.01% aqueous solution of poly-L-lysine (Mr 300 000; Sigma) and then processed for staining with hematoxylin and eosin. Moreover, the number of microabscesses in the liver were counted by light microscopy and areas of the microabscesses were measured by using image-analyzing software NIH image. Ten random fields from five mice livers per group were estimated.

2.8. In situ detection of DNA fragmentation

Apoptotic cells on sections of mice livers were detected with Apop Tag (Oncor, Gaitherburg, MD, USA), an in situ apoptosis detection kit. In brief, sections prepared as described above were incubated with terminal transferase and digoxigenin-labelled-dUTP. Sections were then treated with anti-digoxigenin mAb conjugated to horseradish peroxidase. The slides were developed with peroxidase substrate and counterstained with methylene green.

2.9. Statistical evaluation of the data

Data were expressed as mean ± S.D. and the Mann–Whitney’s U-test was used to determine the differences in bacterial counts, numbers and areas of microabscesses in the organs between the control and experimental groups. Each experiment was repeated at least three times and accepted as valid only when the trials showed similar results.

3. Results

3.1. Induction of DNA fragmentation in the liver and spleen during a lethal infection with L. monocytogenes

When ddY mice were infected i.v. with 5×10⁶ cfu of L. monocytogenes, they died by day 4 of infection [18]. Bacterial growth had increased up to day 3 in both organs (Fig. 1A). Serum AST and ALT levels were also elevated on days 1 and 2 of infection (Fig. 1B). Moreover, DNA fragmentation was observed in the spleen of infected mice from day 1 through day 3, whereas no DNA fragmentation was observed in the liver from day 1 through day 3 (Fig. 1C). No DNA fragmentation was detected in either organ of the uninfected mice.

3.2. Effect of endogenous cytokines on DNA fragmentation in the liver

Our previous study [18] showed that higher titers of endogenous cytokines, including TNF-α, IFN-γ, IL-4, IL-6 and IL-10, are produced in the bloodstream and organs of mice with a lethal infection of L. monocytogenes, compared to a nonlethal infection. Therefore, the effect of in vivo administration of anti-cytokine mAbs on DNA fragmentation in the liver of L. monocytogenes-infected mice was investigated. ddY mice were injected i.v. with mAbs against mouse TNF-α, IFN-γ, IL-4, IL-6 or IL-10, or NRG as the control, 1 h before infection with 5×10⁶ cfu of L. monocytogenes, and bacterial numbers (Fig. 2A) and DNA fragmentation (Fig. 2B) in the liver
were estimated 48 h later. DNA fragmentation in the liver was observed in anti-TNF-α mAb-treated mice and anti-IL-6 mAb-treated animals in which bacterial numbers were significantly higher than in NRG-treated mice (P < 0.01), whereas fragmented DNA was not detected in anti-IFN-γ mAb-, anti-IL-4 mAb-, anti-IL-10 mAb- or NRG-treated mice in which bacterial growth was not augmented by the treatments. On the other hand, DNA fragmentation was observed in the spleen of infected animals irrespective of any anti-cytokine mAb treatments (data not shown).

3.3. Induction of DNA fragmentation in the liver of IFN-γ- or TNF-α-deficient mice during a lethal infection with *L. monocytogenes*

Both TNF-α and IFN-γ are known to play critical roles in antilisterial resistance [19–22]. Fig. 2 showed that anti-IFN-γ mAb treatment failed to affect the bacterial numbers and the induction of DNA fragmentation in the liver of *L. monocytogenes*-infected mice. It is possible that these results arose from the limited ability of anti-IFN-γ mAb used herein. Therefore, we investigated the bacterial growth and DNA fragmentation in the liver of IFN-γ−/− mice and TNF-α−/− mice postinfection. The gene-knockout mice and C57BL/6 mice were infected i.v. with 5 × 10⁶ cfu of *L. monocytogenes* and the number of bacterial cells (Fig. 3A) and DNA fragmentation (Fig. 3B) in the liver of infected mice were investigated 48 h later. Bacterial numbers of *L. monocytogenes* in the liver of IFN-γ−/− mice and TNF-α−/− mice were elevated significantly from those of C57BL/6 mice (P < 0.01). DNA fragmentation was observed in TNF-α−/− mice but not in IFN-γ−/− mice, which was consistent with the results obtained from the corresponding anti-cytokine mAb-injected mice as shown in Fig. 2B.

Table 1

<table>
<thead>
<tr>
<th>Injected with:</th>
<th>No. of microabscesses field⁻¹ (×40)</th>
<th>Microabscess size (μm²) *</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRG</td>
<td>11.8 ± 1.9</td>
<td>5099.3 ± 2268.5</td>
</tr>
<tr>
<td>Anti-TNF-α mAb</td>
<td>15.3 ± 3.9</td>
<td>19826.1 ± 9833.3</td>
</tr>
<tr>
<td>Anti-IL-6 mAb</td>
<td>9.8 ± 1.7</td>
<td>17796.0 ± 3389.0</td>
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* Mice were injected i.v. with anti-TNF-α mAb, anti-IL-6 mAb or NRG 1 h before infection with 5 × 10⁶ cfu of *L. monocytogenes* 1684 strain and the livers were removed 48 h later.

* Sections of liver from the mice were stained with hematoxylin and eosin and numbers of microabscesses per field were counted under a light microscopy at ×40. Each result represents the mean ± S.D. for a group of ten random fields of sections obtained from five mice.

* Areas of the microabscesses were measured by using image analyzing software NIH image. Each result represents the mean ± S.D. for a group of ten random fields of sections from five mice livers per group.

* This value is significantly different from the value for the NRG-treated group (P < 0.01). The results were reproduced in three repeated experiments.
3.4. Detection of apoptotic cells by in situ TUNEL staining in the livers

Mice were injected with anti-TNF-α mAb, anti-IL-6 mAb, or NRG 1 h before infection with $5 \times 10^6$ cfu of *L. monocytogenes* and the livers were removed 48 h later. Normal livers were also removed from uninfected mice. Microabscesses, which consisted of infiltrated polymorphonuclear cells (PMN), macrophages and lymphocytes, were scattered in all of the livers of *L. monocytogenes*-infected mice. Typical histology of the liver from NRG-, anti-TNF-α mAb- and anti-IL-6 mAb-treated mice are shown in Fig. 4. Moreover, the number and area of microabscesses in the livers of each group were calculated (Table 1). The number of microabscesses was not significantly different among the three groups, whereas the area of
microabscesses was significantly greater in the liver of anti-TNF-α mAb- or anti-IL-6 mAb-treated mice, compared with the NRG-treated group (P < 0.01). Next, DNA fragmentation in the liver of L. monocytogenes-infected mice was detected by in situ TUNEL staining. Typical pictures of liver from NRG- or anti-TNF-α mAb- and anti-IL-6 mAb-treated mice are shown in Fig. 4. TUNEL-positive cells were scarcely detected in the liver of NRG-treated mice or in those of uninfected mice (data not shown). In contrast, apoptotic cells were detected in the microabscesses in the liver of anti-TNF-α mAb-treated mice and anti-IL-6 mAb-treated mice. In both groups, the TUNEL-positive cells consisted of infiltrated cells such as PMN. TUNEL-positive hepatocytes were also detected at the edge of microabscesses in the liver of anti-TNF-α mAb-treated mice and anti-IL-6 mAb-treated mice.

4. Discussion

The liver is known to be a principle site of bacterial replication during L. monocytogenes infection in mice [5,6]; liver injury has been observed with severe infection [7], and apoptotic hepatocytes have reportedly been observed [8]. Therefore, we investigated whether liver apoptosis may be involved in lethality caused by L. monocytogenes infection. However, this study showed that liver apoptosis may not be essential in the lethality. We further investigated the regulation of apoptosis by endogenous cytokines during infection. We demonstrated that endogenous TNF-α and IL-6 appear to protect mice from liver apoptosis, whereas IFN-γ does not seem to be a major factor in the regulation of liver apoptosis.

Unanue et al. [8,25] reported that L. monocytogenes infection induces apoptosis of infected hepatocytes in liver and lymphocytes in spleen and lymph nodes. In this study, when mice were infected with a lethal dose of L. monocytogenes cells, apparent DNA fragmentation was not observed in the livers (Figs. 1 and 4). The reason for this contradictory result is not clear. In contrast, liver apoptosis was obviously detected in mice which had been treated with mAb against TNF-α or IL-6 (Figs. 2 and 4) or in TNF-α−/− mice (Fig. 3). On the other hand, serum AST and ALT are parenchymal cell enzymes commonly used as markers of liver cell necrosis. In this study, the levels of both enzymes were increased after infection (Fig. 1B), suggesting that liver necrosis is induced by infection.

TNF-α is well known to induce apoptosis in various types of cells [26] and the apoptotic death of hepatocytes is reportedly regulated by TNF-α [11,15]. Alternatively, TNF-α reportedly inhibits apoptosis by inducing nuclear factor-κB activation [27]. In the present study, liver apoptosis induced by L. monocytogenes infection was augmented in anti-TNF-α mAb-treated mice (Figs. 2 and 4) and in TNF-α−/− mice (Fig. 3). The number of L. monocytogenes cells in the liver was markedly increased in anti-TNF-α mAb-treated mice (Fig. 2) and in TNF-α−/− mice (Fig. 3). Moreover, when anti-TNF-α mAb-treated mice were infected with listeriolysin O gene-defective avirulent mutant DP-L2161 [28], which does not proliferate intracellularly, apoptosis was not detected in the liver (data not shown). In this study, the augmentation of liver apoptosis and a marked increase in bacterial numbers in the liver were also observed in anti-IL-6 mAb-treated mice (Fig. 2). These results seem to indicate the possibility that excess bacterial growth may cause apoptosis. However, the results obtained from anti-IFN-γ mAb-treated mice and IFN-γ−/− mice tend to contradict this possibility.

IFN-γ is a major factor in antilisterial resistance [19,20]. IFN-γ reportedly modulates apoptosis such as hepatitis induced by LPS in P. acnes- or BCG-sensitized mice [12,13], or by Con A in mice [14]. In this study, the augmentation of DNA fragmentation and bacterial growth in the liver was not observed in anti-IFN-γ mAb-treated mice (Fig. 2). In a previous study, clone R4-6A2-derived anti-IFN-γ mAb was reported to show less neutralizing activity in vivo [29]. We presumed that apoptosis may not be augmented by the failure of inhibiting antilisterial resistance by mAbs, because no augmentation of DNA fragmentation was observed in the liver of IFN-γ−/− mice, irrespective of the marked increase in bacterial numbers in the liver (Fig. 4). This indicates that IFN-γ may not be involved in the regulation of liver apoptosis in L. monocytogenes infection and that augmentation of liver apoptosis in TNF-α-deficient mice and IL-6-deficient mice may not merely be due to the increase in bacterial growth in these organs.

It has been reported that L. monocytogenes infection induces apoptosis in hepatocytes [8], lymphocytes [25] and dendritic cells [30], but not macrophages [31] in vitro and in vivo. Lymphocytes are the major apoptotic cells in spleen [25]. We presumed that most apoptotic cells would be lymphocytes which infiltrated to the liver in TNF-α- or IL-6-deficient mice. However, the TUNEL-positive cells were mainly PMN and partly hepatocytes in the liver of these animals (Fig. 4) and DNA fragmentation was not observed in the mononuclear cell fraction (unpublished observation). It is presumed that TNF-α and IL-6 may play an important role in protecting infiltrating neutrophils from apoptosis.

In summary, TNF-α and IL-6, but not IFN-γ, may play an important role in protecting the liver from apoptosis in lethal L. monocytogenes infection. These three cytokines are known to be critical in antilisterial resistance [19–22]. However, the involvement of IFN-γ in liver apoptosis seems to be inconsistent with that of TNF-α and IL-6 in lethal L. monocytogenes infection. We are now studying the mechanism of different roles of TNF-α and IFN-γ in liver apoptosis during L. monocytogenes infection.
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References


